Clustered Regularly Interspaced Short Palindromic Repeats-Associated Protein 9 in *Pichia pastoris*: The Combination of Gene Editing and Biological Manufacturing

YUCHEN XIANG* AND XIANFENG DIING

Department of Medical Sciences, School of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou 310018, China

The clustered regularly interspaced short palindromic repeats-associated protein 9 technology stands out as the paramount gene-editing tool owing to its unparalleled efficiency and precision. It has rapidly been applied to various biological systems, including bacteria, plants and yeasts. *Pichia pastoris* (now known as *Komagataella phaffii*) is a commonly used industrial microorganism with advantages such as rapid growth, high-density culture, robust expression of exogenous proteins, with its simplicity in genomic manipulation, clustered regularly interspaced short palindromic repeats-associated protein 9 renders itself as an optimal platform for synthesizing recombinant proteins, enzymes and various biologics. The application of clustered regularly interspaced short palindromic repeats-associated protein 9 in *Pichia pastoris* focuses on gene editing and bio manufacturing, enabling precise modifications of its genome to enhance metabolic pathways and improve product quality. Recent advancements have demonstrated significant progress utilizing clustered regularly interspaced short palindromic repeats-associated protein 9 for metabolic engineering and protein manipulation in *Pichia pastoris*, substantially increasing the yield of target metabolites and recombinant proteins. Despite these advances, technical challenges such as off-target effects and unstable editing efficiency remain. Therefore, further optimization of the clustered regularly interspaced short palindromic repeats-associated protein 9 system, development of new Cas variants, and improvement in guide ribonucleic acid design are current research hotspots and challenges. This review systematically summarizes the progress of clustered regularly interspaced short palindromic repeats-associated protein 9 application in *Pichia pastoris*, focusing on specific application examples and technology optimization strategies in gene editing and bio manufacturing. It also discusses the current challenges and future research directions. The aim is to provide researchers with a comprehensive and in-depth knowledge framework to promote the further development and application of this technology in bio manufacturing, stimulate more innovative research ideas, and bring new breakthroughs in the genetic engineering and bio manufacturing of *Pichia pastoris*.

Key words: Clustered regularly interspaced short palindromic repeats-associated protein 9, gene editing, *Pichia pastoris*, bio manufacturing

Gene editing has undergone rapid development in the past few decades, and Clustered Regularly Interspaced Short Palindromic Repeats-Associated Protein 9 (CRISPR/Cas9) has become one of the most influential gene editing tools due to its high efficiency, precision and relatively simple operation[1]. Since the CRISPR/Cas9 system was first adapted for use in mammalian cells in 2012, this system has rapidly expanded to a variety of biological systems[2,3]. The core components of CRISPR/Cas9 include the Cas9 nuclease and guide Ribonucleic Acid (gRNA), which work together to act on specific Deoxyribonucleic Acid (DNA) sequences to induce double-stranded breaks, allowing for the targeted editing of genes[4]. Due to its efficiency and flexibility, CRISPR-Cas9 shows great potential in areas such as basic research, disease treatment and bio manufacturing[5].

Pichia pastoris (*P. pastoris*), now renamed *Komagataella phaffii*[6], is a commonly used methanolic nutritive yeast that is important in bio manufacturing[7]. Its advantages include rapid growth, high cell density culture, robust exogenous protein

***Address for correspondence**
E-mail: 2021332864035@mails.zstu.edu.cn

expression and easy genomic manipulation[8]. These properties of *P. pastoris* make it an ideal host for the production of recombinant proteins, enzymes and other biologics[9]. Employing CRISPR/Cas9 offers a superiorly efficient and accurate method for editing the genome of *Pichia* yeast, significantly increasing the speed and flexibility of genetic modification[10].

The application of the CRISPR/Cas9 in *P. pastoris* focuses on two aspects; gene editing and bio manufacturing. Through gene editing, precise modifications of the Picot yeast genome can be realized, including gene knockout, knock-in, mutation and regulation[11]. Emerging gene editing methods provide new means to study the physiological functions, optimize the metabolic pathways and improve the bio manufacturing capacity of *P. pastoris*. In bio manufacturing, *P. pastoris* is widely used as a good bioreactor for the production of a variety of recombinant proteins, including pharmaceutical proteins, industrial enzymes and vaccines[12]. The application of CRISPR/Cas9 has greatly enhanced the productivity and product quality of *P. pastoris* in these areas.

Over the past few years, numerous research endeavors have documented the effective utilization of CRISPR/Cas9 within *P. pastoris*. Protein engineering of *P. pastoris* using CRISPR/Cas9 can optimize its protein expression system and improve the yield and quality of recombinant proteins[13]. However, despite the remarkable progress in the application of CRISPR/Cas9 in *P. pastoris*, there are still some technical challenges, such as off-target effects and unstable editing efficiency[14]. Therefore, further optimization of the CRISPR/Cas9 system, development of new Cas variants and improvement of gRNA design are the hotspots and difficulties of current research.

This review aims to systematically summarize the progress of CRISPR/Cas9 application in *P. pastoris*, focusing on its specific application examples and technology optimization strategies in gene editing and bio manufacturing. First, we will introduce the principles and advantages of CRISPR/Cas9 technology, then discuss in detail its specific applications and optimization methods in *P. pastoris* genome editing. Then, we will overview examples of CRISPR/Cas9 applications in *P. pastoris* bio manufacturing, including recent advances in metabolic engineering and protein engineering. Finally, we'll examine the present hurdles facing the implementation of CRISPR/Cas9 technology in *P. pastoris* and anticipate forthcoming research pathways and prospects for advancement. Through this review, we hope to provide researchers with a comprehensive and in-depth knowledge framework of the application of CRISPR/Cas9 in *P. pastoris*, and to promote the further development and application of this technology in the field of bio manufacturing. The review article not only summarizes the current research results, but also proposes future research directions, which are expected to stimulate more innovative research ideas and bring new breakthroughs in genetic engineering and biomanufacturing of *P. pastoris*.

MECHANISMS OF CRISPR/CAS9 WORK IN *P. pastoris*

CRISPR/Cas9 can be used for site-specific cleavage in *P. pastoris* by designing gRNAs based on the target DNA sequence. gRNAs are composed of two parts; CRISPR RNA (crRNA), which is responsible for recognizing and binding to the target DNA sequence, and a trans-activating CRISPR RNA (tracrRNA), which binds to the crRNA and directs the Cas9 nuclease[15]. Genes encoding gRNA and Cas9 proteins are inserted into an expression vector. These vectors are usually free plasmids capable of replication and expression in *P. pastoris* cells[16]. Expression vectors were introduced into *P. pastoris* cells by electroporation[17]. After transformation, cells express gRNA and Cas9 proteins. In *P. pastoris* cells, the gRNA directs the Cas9 protein to target DNA sequences that are complementary to the gRNA. The target sequence usually has a short Protospacer Adjacent Motif (PAM) sequence upstream, which Cas9 recognizes and binds to[18]. As soon as the gRNA binds to the target DNA sequence, the Cas9 protein triggers a Double-Strand Break (DSB) at the specific site of the target sequence.

After DSB occurs, *P. pastoris* cells undergo DNA repair, and in the absence of a template, *P. pastoris* genes are inactivated due to random deletions or insertions of bases after being cut by CRISPR/Cas9, which triggers the Non-Homologous End Joining (NHEJ) repair mechanism in *P. pastoris*[19]. Although NHEJ repair is very hard to predict, high-fidelity knockdown of short overlapping sequences (30 bq) is currently possible by amplification and Sanger sequencing to screen target sites for insertion of deletion mutations and to confirm the formation of early stop codons[20]. Liao *et al.*[21] found that the yeast pGAP or pCYC1 promoter-driven reporter gene

eGFP exhibited strong repression (>70 %) with up to ~3.5-fold activation. Gene editing by using RNA Pol III promoter-driven single guide RNA (sgRNA) expression. Rapid recovery of selectable markers using eliminable free plasmids enabled knockdown efficiencies of up to 100 %. In general, *P. pastoris* has a high probability of developing NHEJ, which means that CRISPR/Cas9 can be targeted to knock out a specific gene on the *P. pastoris* genome, and the function of the gene can be determined after screening and culture. Therefore, CRISPR/Cas9 can also be applied to the functional genomics of *P. pastoris* in the future. In addition, new defective types of *P. pastoris* can also be realized by targeted gene knockout using CRISPR/Cas9[22].

If a donor DNA fragment containing the desired modification is provided, the cell can be precisely repaired using the Homologous Recombination (HR) mechanism, which enables knock-in or specific gene editing cells to recognize the donor DNA (donor DNA) containing the homologous arm[23]. Donor DNA is inserted at the target location or replaced with a specific sequence after the design is complete. The ends of the donor DNA contain sequences homologous to the ends of the DSB, and these homologous arms are usually between tens and hundreds of base pairs in length. The single-stranded end of the damaged DNA intrudes into the homologous sequence of the donor DNA, forming a D-loop. The donor DNA will serve as a template and new DNA strands are synthesized at the break position. Eventually it is Holliday junctions that are formed and untwisted. During the repair process, temporary crossover structures are formed and these are eventually unwound, restoring double-stranded DNA. This method has been widely used in *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Yarrowia lipolytica*, and the process is similar for the more closely related *P. pastoris* to both when performing HR[24].

P. pastoris is a commonly used industrial microorganism with a robust expression system and genetic stability to secrete proteins at high yields[25]. CRISPR/Cas9 can be used to optimize *P. pastoris* metabolic pathways and increase the yield of some products. For example, editing genes associated with metabolic pathways could increase the yield of biofuels, pharmaceutical intermediates or other high value-added compounds. For example, editing genes associated with metabolic pathways can increase the production of biofuels, pharmaceutical intermediates or other high value-added compounds. CRISPR/Cas9 can be used to optimize *P. pastoris* metabolic pathways and increase the yield of some products. For example, editing genes associated with metabolic pathways can increase the yield of biofuels, pharmaceutical intermediates or other high value-added compounds. *P. pastoris* can tolerate high concentrations of 3-methyl-1-butanol, which makes it well suited for use in the production of isoamyl alcohol (3-methyl-1-butanol) as biodiesel. Down-regulation of by-product ethanol production using the CRISPR/Cas9 system resulted in a strain capable of producing 3M1B at a titer of 191.0±9.6 mg/l, which is the highest titer reported to date in an unconventional yeast[26].

In addition to CRISPR/Cas9, other members of the CRISPR family have shown significant promise in gene editing *P. pastoris*. Smirnov *et al.*[27] tested several codon optimizations of the MAD7 gene in conjunction with a variety of NLS and gRNA scaffolding sequences, demonstrating that CRISPR/MAD7 can also be used for *P. pastoris* gene editing. However, gene knockdown of *P. pastoris* by CRISPR/MAD7 is only 23 %-65 % as efficient as Cas9, and still requires continuous modification and improvement. CRISPR/dCas9 has also been shown to be used to target repression of promoters eGFP and AOXI, leading to the repression of fatty acyl coenzyme A synthetase genes (FAA1 and FAA2). Meanwhile, CRISPR/dCas9 showed strong inhibition of eGFP in the experiment and the highest efficiency could reach 85 %[28]. For their part, Baumschabl *et al.*[29] found that dCas9 can be fused to trans activator domains *via* RNA scaffolds to regulate gene expression when targeting gene promoter regions, which occurs when methanol or glucose are used as carbon sources.

Currently, other CRISPR family members, such as CRISPR/Cas12a (Cpf1), CRISPR/Cas13, CRISPR/dCas9 (inactivated Cas9), CRISPR/Cas3 and CRISPR/Cas9 variants, are also showing promising results in microbial gene editing applications[30]. In the future, as CRISPR technology continues to improve and update, more new CRISPR systems will surely be applied to gene editing in *P. pastoris*.

REDIRECTING *P. pastoris* METABOLIC FLOW BY CRISPR/CAS9

Redirecting metabolic flux refers to altering the flow of metabolic pathways within a cell by means of genetic engineering in order to optimize the synthesis of specific metabolites or reduce the production of

byproducts[31]. This approach is particularly important in metabolic engineering, where it can be a good way to increase the efficiency of microbial or other biological systems in producing specific compounds. CRISPR/Cas9 can be used to knock out competitive pathway genes for metabolic pathway optimization. This process is mainly realized through the NHEJ pathway[32]. If key genes in metabolic pathways that compete with target product synthesis for substrate or energy are deleted or inhibited, the by-product synthesis pathway in *P. pastoris* will be inhibited. Thus *P. pastoris* can use more substrate and energy for target product synthesis[33].

P. pastoris has excellent compatibility with exogenous genes. Unlike model organisms such as *S. cerevisiae* and *Escherichia coli* (*E. coli*), the mechanism of protein expression in *P. pastoris* is very similar to that of eukaryotes, especially mammalian cells[9]. *P. pastoris* has a powerful secretion mechanism that can efficiently secrete a wide range of recombinant proteins and a protein glycosylation pattern that is also closer to that of humans compared to that of *S. cerevisiae*. This makes it an ideal protein expression system, especially when high levels of secretion are required[34].

Therefore, *P. pastoris* has great potential for the production of biofuels, pharmaceutical intermediates and other high value-added compounds[35]. Importing crtE, crtB, and crtI into *P. pastoris* GS115 by CRISPR/Cas12a establishes the heterologous lycopene pathway in *P. pastoris* and is also considered to be the most efficient pathway for lycopene expression in *P. pastoris* at present in the middle[30]. By designing modular, reliable gRNA-expressing host-specific cassettes, CRISPR/Cas9 can achieve 95 % editing efficiency in *P. pastoris*, resulting in the expression of human fibrinogen (K3), a humanized product[36].

The protein expression system of *P. pastoris* can be optimized by gene editing with CRISPR/Cas9, including knocking out genes that inhibit protein expression or inserting enhancer elements to increase the efficiency of exogenous protein expression. Liu *et al.*[37] used CRISPR/Cas9 as a template and designed CRISPRi and CRISPRiD for repression regulation and activation regulation, respectively, to produce α-amylase using *P. pastoris* SynPic-R as a bioreactor. Also they pointed out that SynPic-R was more efficient than GS115 in expressing α-amylase. As a technologically sophisticated vector, *P. pastoris* will be used to test exogenous genes from other species. Zhang *et al.*[38] successfully used CRISPR/Cas to edit the gene of *P. pastoris* to enable it to synthesize the CP protein of *Clostridium mangostanus* (CmCP).

It is foreseen that in *P. pastoris*, by knocking out key genes in the ethanol fermentation pathway (e.g. Alcohol Dehydrogenase I (ADH1), Pyruvate Decarboxylase 1 (PDC1)) and overexpressing key enzymes in the fatty acid synthesis pathway (eg. Acetyl-CoA Carboxylase 1 (ACC1)), more carbon sources can be used for fatty acid synthesis, thus increasing the yield of biodiesel[39]. Similar to *S. cerevisiae*, ADH1 in *P. pastoris* catalyzes the acetaldehyde reduction reaction to ethanol in the presence of cofactors, showing the potential to reduce the acetaldehyde content in wine[40]. Integration of multiple heterologous genes into *P. pastoris* cells to synthesize 2,3-Butanediol (BDO) in large quantities *via* CRISPR/Cas9 has been shown to be feasible[41]. Besides CRISPR/Cas offers the possibility of designing and introducing new metabolic pathways. Gao *et al.*[42] used a series of gRNA plasmids (for single-site, two-site and three-site integration) and a donor plasmid (containing homology arms for integration and promoters and terminators to drive the expression of heterologous genes) to recombine the *P. pastoris* GS115 to enable the synthesis of BDO, β-carotene, zeaxanthin and astaxanthin using methanol as a carbon source. *P. pastoris* can also be used as a bioreactor for the production of terpenoids, polyketides, flavonoids and especially α-sandalene (a bicyclic sesquiterpene). Combined with media optimization and bioprocess engineering, the best strain (STE-9) produced titers of up to 829.8±70.6 mg/l in shaker flasks, 4.4±0.3 g/l in batch fermenter and 4.4±0.3 g/l in refilled batch fermenter, respectively and 21.5±1.6 g/l of α-sandalene, respectively. These represent the highest α-sandalene yields ever reported, highlighting the advantages of *P. pastoris* in the production of terpenoids and other natural products[43].

PROBLEMS AND CHALLENGES IN CRISPR/CAS9 APPLICATION FOR GENE EDITING IN *P. pastoris* CEREVISIAE

S. cerevisiae is an important carrier for traditional biosynthesis with low production cost, mature technology and simple operation. *P. pastoris* is able to grow at high density in the fermenter and the highest cell density can reach 130 g/l stem cell weight, which is very favorable for industrial production[44]. Moreover, *P. pastoris* is more receptive to exogenous

genes, and the protein expression mechanism of *P. pastoris* is more similar to that of human body[45]. *P. pastoris* has a unique methanol-induced expression. The AOX1 promoter of *P. pastoris* is strongly induced in the presence of methanol, which enables efficient and controlled exogenous protein expression. In industrial practice, *P. pastoris* is also favored by researchers for its low fermentation by-products and high tolerance to stressful environments[42].

P. pastoris has a much lower HR efficiency compared to commonly used *S. cerevisiae*. This may be because the homology between the donor DNA and the target site is not high enough or the homology arm length is insufficient. It may also be because the intracellular environment of *Pichia* yeast may be unfavorable to HR, limiting the activity of homologous recombinase or the stability of donor DNA. Under the effect of multiple factors, Picros yeast prefers to use the NHEJ mechanism rather than the HR mechanism in repairing DNA and DSBs. However, NHEJ is a fast but less precise repair method and is mainly used for gene knockout rather than gene editing.

Thus, the use of CRISPR/Cas9 for gene editing in *P. pastoris* remains challenging. Deletions of *P. pastoris* target gene disruptions are usually inserted randomly into the genome through ectopic integration (correct targeting rate of <1 %-30 %), requiring laborious screening procedures to identify the correct knockout strains[46]. This has caused great difficulties for many research teams. The efficiency of *P. pastoris* HR can be effectively improved by developing a novel CRISPR/Cas9 system with High Editing Efficiency and Recoverable dystrophy-Selective Markers (HiEE-ReSM)[47]. A NHEJ defective strain (Δku70) was constructed based on the CRISPR/Cas9 gene defective method. It was then used as a parental strain for multilocus gene integration. Ten gRNA targets were designed within 100 bp upstream of the promoter or downstream of the terminator, and then tested and confirmed as suitable single-locus integration sites using eGFP reporter genes[48]. Nucleic acid exonuclease binding to Cas9 enhances homologous recombination in *P. pastoris* cells, and endogenous nucleic acid exonucleases MRE11 and EXO1 have the highest positivity in FAA1 seamless deletion, and MRE11 has the highest positivity for fusion to the C-terminus of CAS9, with a relatively high number of clones[49].

Some researchers have also broken the inefficient HR mechanism of *P. pastoris* itself by introducing the HR mechanism of *S. cerevisiae* and using a short (40 bp) homology arm in *P. pastoris* to achieve multiple genome integration[50]. Not only that, but improving HR efficiency in *P. pastoris* by means of disrupting the NHEJ machinery has been the subject of heated discussion. Dalvie *et al.*[36] have attacked this problem through an alternative approach. Their sequencing-based strategy was used to design host-specific cassettes for modular, reliable and guided RNA expression. Using this strategy, they successfully achieved up to 95 % gene editing efficiency in *P. pastoris*.

Gene editing of *P. pastoris* with CRISPR/Cas9 also suffers from off-target problems. This problem can be effectively avoided by using a high-fidelity Cas9 enzyme (eSpCas9, SpCas9-HF1), which enables more precise editing. Designing primers and RNAs with high specificity is also considered a feasible approach. Along with the development of bioinformatics technology, using bioinformatics tools to predict potential off-target sites to avoid selecting primers and RNA fragments similar to off-target sites is also worth considering. Currently, the off-target problem of CRISPR/Cas9 has been better solved in human cells, bacteria and so on[51,52].

Glycosylation can generally be divided into O-mannosylation and N-glycosylation, both of which are highly conserved in biological evolution. The O-glycosylation of *P. pastoris* is much simpler compared to mammals, with a maximum of only six mannose residues[53]. N-glycosylation of *P. pastoris* generates mainly sugar chains containing many mannose sugars, containing a sugar molecule not found in mammalian cells, the α-1,3-mannose modification. Humanization of the process of glycosylation of *P. pastoris* by gene editing has now been shown to be feasible, and Bretthauer et al.[54] have begun to produce protein-based drugs by this method many years ago. It is clear that the efficiency of modifying glycosylated genes by the CRISPR/Cas9 system has been considerably higher than several past methods, including ZFN and TALEN[13].

Methanol, as a toxic and flammable hazardous compound, is dangerous during storage and use. The process of using methanol to induce the *P. pastoris* AOXI promoter can be very polluting and environmentally unfriendly. Attempting to get *P. pastoris* to stop using methanol as a carbon source by means of gene editing seems to be difficult, and there are very few teams researching this area. Only

Gassler *et al.*[55] attempted to establish a carbon source fixation pathway similar to the Calvin-Benson-Bassham cycle by modifying the *P. pastoris* genome to free it from methanol dependence and make it an autotrophic strain. However, the carbon source of this method is carbon monoxide, which is still dangerous. The future application of CRISPR/Cas9 technology in *P. pastoris* genome modification should change this situation significantly.

Although CRISPR-based genome editing tools are available for *P. pastoris* there is still a severe shortage of tools and genetic elements currently available for metabolic engineering of *P. pastoris* compared to commonly used model organisms including *S. cerevisiae*, *E. coli* and other microorganisms[56]. Future research will require the development of additional tools such as promoter libraries, UTR sequences, terminator sequences, genomic loci for gene integration, dynamic regulatory tools and other genetic material[57]. With the development of RNA-seq, single-cell analysis, and high-throughput analysis, it has become convenient to develop tools for genetic modification of *P. pastoris*[35].

CONCLUSION

This paper reviews the multiple applications of CRISPR/Cas9 technology in *P. pastoris*, including gene knockout, gene knock-in, metabolic engineering and protein expression optimization. CRISPR technology has demonstrated great advantages in the field of genome editing due to its high efficiency, precision and versatility. *P. pastoris*, as an important biotechnology platform, has been widely used in biopharmaceuticals, industrial enzyme production and food additives due to its rapid growth, simple culture conditions and high protein expression capacity. Through the application of CRISPR technology, significant progress has been made in the research and application of *P. pastoris*, including metabolic pathway optimization and redirection of metabolic flow.

In the future, the combination of CRISPR technology with synthetic biology, metabolomics and other emerging biotechnologies will further expand the research and application fields of *P. pastoris*. Improvement of CRISPR systems to enhance editing efficiency, reduce off-target effects and develop novel CRISPR systems will be important research directions in the future. In addition, the application of CRISPR technology in optimizing the production process of *P. pastoris* and developing new biological products is promising. The higher NHEJ efficiency and lower HR efficiency of *P. pastoris* make CRISPR/Cas9 suitable for performing functional genomics research and phenotypic screening.

However, current technologies still face challenges in gene editing efficiency, off-target effects, and biosafety, so CRISPR/Cas has been used far less in *Microsporum* than in *S. cerevisiae* and *E. coli*. Future research needs to focus on solving these problems and advancing the widespread application of CRISPR technology in *P. pastoris* through interdisciplinary and international collaborations to realize its great potential in bio manufacturing and basic research.

Conflict of interests:

The authors declared no conflict of interests.

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